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Involvement of antizyme-like regulatory protein in polyamine-caused repression of ornithine decarboxylase in insect-derived *Trichoplusia ni* 5 cells

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Abstract

Addition of spermidine to culture medium of insect cells, *Trichoplusia ni* 5, at a low cellular density suppressed ornithine decarboxylase (ODC; EC 4.1.1.17) activity and induced ODC inhibitory activity. The inhibitory factor was non-dialyzable, temperature-sensitive, and could reversibly form an inactive complex with ODC. It showed a time-independent and non-stoichiometric pattern of inhibition. Upon addition of spermidine to cultured cells with preinduced ODC, the enzyme decayed more rapidly than after addition of cycloheximide. These data strongly suggested that ODC of Tn5 cells is under negative feedback control by polyamines, in which an antizyme-like regulatory protein plays an essential role. © 1997 Elsevier Science B.V.

Keywords: Ornithine decarboxylase; Antizyme; Polyamine; Insect; *Trichoplusia ni* 5 cell

1. Introduction

Ornithine decarboxylase (ODC; EC 4.1.1.17), a key enzyme in polyamine biosynthesis [1], turns over at variable rates with a half life ranging from several minutes to over 1 h [2]. Mammalian ODC is rapidly induced upon various growth and differentiation stimuli [3,4], and is under negative feedback control by polyamines, in which a unique regulatory protein named antizyme plays an essential role [5]. Induced by polyamine-caused translational frameshifting [6], antizyme binds to ODC, not only inhibiting its activity but also accelerating its degradation by the 26S proteasome [5,7]. Antizyme also suppresses cellular uptake of polyamines [8,9]. Thus antizyme plays an important role to effectively prevent excess accumulation of cellular polyamines [10].

Antizyme-mediated ODC regulation has been studied mainly in vertebrates. In lower eukaryotes, ODC appears to be regulated in various different manners [11]. In protozoan, we recently reported that antizyme is not present and ODC is not destabilized by polyamines in *Tetrahymena* [12].

In various species of insects, ODC activity and cellular polyamine levels fluctuate with growth and metamorphosis processes during their life cycles [13–19]. ODC is also induced by hormonal stimulation and injury treatment (puncture to the body) [20,21]. In *Drosophila melanogaster*, ODC gene and cDNA

Abbreviations: Act D, actinomycin D; DFMO, α-difluoromethylornithine; DTT, dithiothreitol; ODC, ornithine decarboxylase; PLP, pyridoxal phosphate; PUT, putrescine; SPD, spermidine; SPM, spermine; Tn, *Trichoplusia ni*

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have been isolated [22]. It has not been reported in insect, however, whether ODC is under negative feedback control by polyamines and, if yes, whether antizyme is involved in it. In the present study we examined these problems in insect-derived *Trichoplusia ni* 5 cells, widely used as host cells for baculovirus.

2. Materials and Methods

2.1. Materials

Trichoplusia ni 5 (Tn5) cells were kindly provided by Dr. Matsuura (National Institute of Health, Tokyo, Japan). L-[1-^{14}C]Ornithine hydrochloride was obtained from Moravek Biochemicals (California, USA). Culture medium (Sf-900 II SFM) was obtained from Gibco BRL (New York, USA). α-Difluoromethylornithine (DFMO) was kindly provided by Dr. P.P. McCann, (Marion Merrell Dow Research Institute, Cincinnati, OH, USA). Recombinant rat antizyme was prepared from rat antizyme Z1 cDNA [23] using maltose binding protein expression vector system (New England Biolabs).

2.2. Cell culture

Tn5 cells were used in all experiments. The cells were cultured in polystyrene tissue culture dishes (Corning, New York, USA or Iwaki Glass, Tokyo, Japan) or Erlenmeyer flasks at 27°C in Sf-900 II SFM medium.

2.3. Assay of ODC activity

Cells were collected by pipetting and scraping with a rubber policeman, and centrifuged at 1000 rpm for 1.5 min. The pellet was resuspended and washed twice with 50 mM Tris-HCl buffer (pH 7.4), containing 0.15 M sucrose, 2 mM dithiothreitol (DTT) and 0.01% Tween 80 (buffer A), and then frozen at −80°C. When needed, the cell pellets were thawed and homogenized with buffer A using a Dounce-type all glass homogenizer. The homogenate was centrifuged at $30\,000 \times g$ for 60 min and the supernatant was used as a crude extract for enzyme and protein assays, as described previously [24]. One unit of the enzyme is defined as the amount catalyzing formation of 1 nmol of CO_2/h at 37°C.

2.4. Induction and assay of ODC inhibitory factor

For the induction of ODC inhibitory factor, cells were inoculated at $1.5 \cdot 10^5$ cells/ml and at the same time spermidine (final 0.7 mM) was added to the 50 ml culture medium in a 500 ml Erlenmeyer flask. After incubation with gentle shaking for 5 days, cells were harvested and the cell extract was prepared and dialyzed extensively against buffer A as described above.

2.5. Preparation of DFMO-ODC

We confirmed that ODC of Tn5 cells could be inactivated by DFMO ($K_i = 1.5$ μM, data not shown). DFMO-ODC was prepared by incubating ODC (33.5 units/mg protein) with DFMO (100 μM) at 37°C for 2 h, and dialyzed extensively against buffer A containing 10 μM pyridoxal phosphate (PLP).

3. Results

We first examined the time course of ODC induction in Tn5 cells and the characteristics of ODC induced. Tn5 cells were grown in Sf-900 II SFM medium to a stationary phase and ODC activity was induced by cell dilution with fresh medium. Tn5 cells reached a stationary phase after 4 or 5 days of transfer. ODC activity markedly increased, reaching a peak at the end of logarithmic growth phase, and then decreased rapidly during the stationary phase (data not shown). The Tn5 ODC had a K_m for ornithine of 0.1 mM (data not shown), which was similar to the K_m of mammalian and yeast ODCs [25]. ODC activity was weakly inhibited by polyamines in vitro (K_i for putrescine, spermidine, and spermine, were 2.5, 1.2, and 0.5 mM, respectively.). One of the features of Tn5 cell ODC is that ODC activity is not proportional to its amount, that is to say, ODC activity is inhibited at low ODC concentration (cf. Fig. 2). This phenomenon is partially removed by addition of final 10% concentration of glycerol to assay mixture. It appears that the coefficient of dimer formation is low at low ODC concentration.

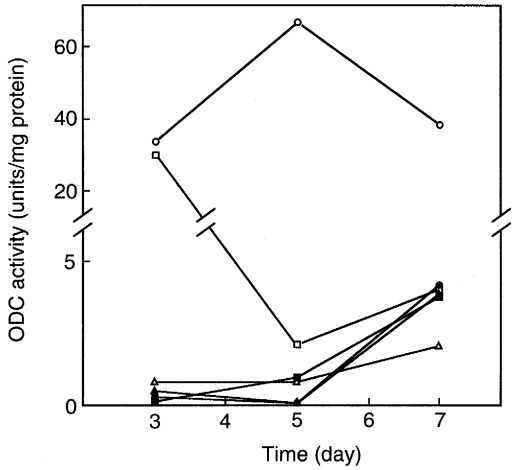

Fig. 1. Repression of ODC induction by polyamines. Confluent cells, which had almost no ODC activity, were diluted to $1.5 \cdot 10^5$ cells/ml with fresh medium containing 1 mM putrescine (□), 10 mM putrescine (△), 0.5 mM spermidine (●), 1 mM spermidine (▲), 0.05 mM spermine (■), no additions (○). Cells were harvested at indicated times and their extracts were assayed for ODC activity as described in Section 2.

We next examined in vivo effects of polyamines on ODC in Tn5 cells. Putrescine, spermidine and spermine repressed ODC induction and induced ODC inhibitory activity (Fig. 1 and Table 1). The inhibitory factor was non-dialyzable and temperature sensitive, indicating that it was a protein. It inhibited ODC in a time-independent manner(data not shown). As shown in Fig. 2, ODC activity was not inhibited equivalently with a fixed amount of the inhibitor. So its inhibition manner was non-stoichiometric. In mammalian cells antizyme can form a reversible complex with ODC, from which active ODC can be released competitively by addition of excess amounts

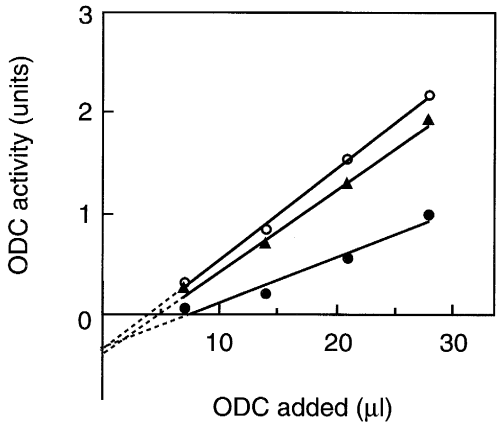

Fig. 2. Inhibitory activity against various amounts of ODC. Different amounts of ODC were assayed in the presence (●) or absence (○) of a fixed amount of the inhibitor. A part of the inhibitor had been heated at 60°C for 20 min (▲).

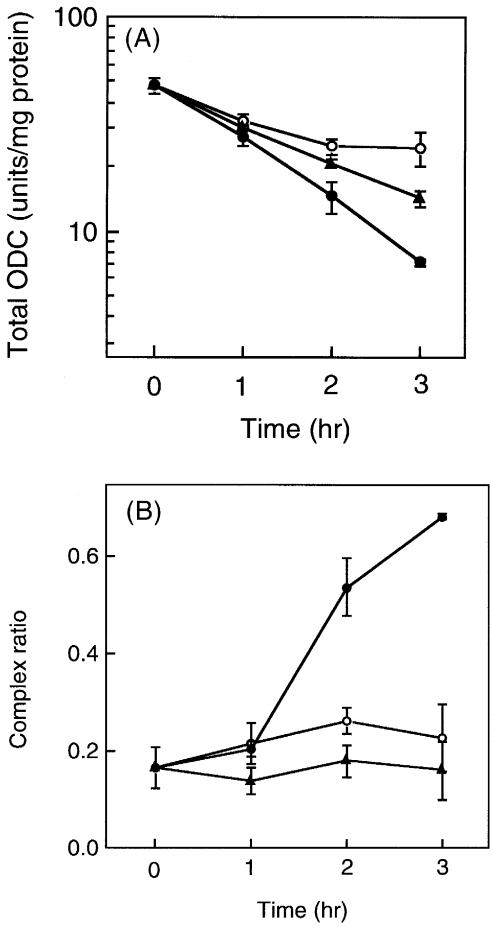

Fig. 3. Decay of total ODC activity (A) and complex ratio (B) in Tn5 cells. Tissue culture dishes of 30 mm diameter were used in this experiment. After 4 days of culture, spermidine (0.2 mM) (●), cycloheximide (100 μg/ml) (○), or both (▲) were added to the culture. Cells were harvested (cell pellets volume was about 100 μl) at indicated times and homogenized with 1 ml buffer A. After centrifugation, 130 μl cell extract was assayed for ODC activity with and without DFMO-ODC (7.56 units) in final 375 μl assay system as described in Section 2. ODC activity was determined in the presence and absence of DFMO-ODC (7.56 units) for total and free ODC activities, respectively. The amount of complex ODC was calculated as the difference between total and free ODC activities. Complex ratio indicates complex ODC/total ODC. Values are means ± S.D. of three experiments except 0 time (four experiments). Similar results were obtained in two other experiments.

Table 1
Induction of ODC inhibitory activity by polyamines

Polyamines (mM)	PUT (10)	SPD (0.5)	SPD (1.0)	SPM (0.05)
ODC inhibitory act. (U/mg protein)	0.44	1.41	2.64	1.09

The extracts of cells cultured for 5 days in the presence of the indicated concentration of polyamines (Fig. 1) were dialyzed against buffer A for more than 2 h twice and overnight. The dialyzed extracts were mixed with 1.75 U of ODC and the mixtures were assayed for ODC activity. One unit of inhibitory factor is defined as the amount inhibiting one unit of ODC in this experiment.

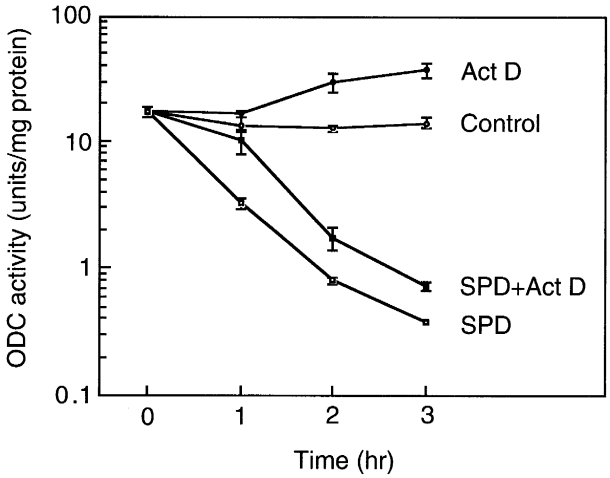

Fig. 4. Effect of actinomycin D on ODC activity in the presence or absence of spermidine in Tn5 cells. Tissue culture dishes of 30 mm diameter were used in this experiment. After 4 days of culture, actinomycin D (1 μg/ml) (△), spermidine (0.2 mM) (●) or both (▲) were added to the culture (○, control). Cells were harvested at indicated times and homogenized with 0.75 ml buffer A. After centrifugation, 65 μl cell extracts were assayed for ODC activity in final 125 μl assay system as described in Section 2. Values are means ± S.D. of three experiments. Similar results were obtained in another experiment.

of DFMO-inactivated ODC, and therefore an increase in ODC activity by this treatment can be taken as the amount of ODC/antizyme complex [26]. In extracts of Tn5 cells that had been cultured with spermidine for 5 days addition of excess amounts of DFMO-ODC reactivated the ODC that had been inactivated by the inhibitory factor (Table 2), indicating that the inhibitory factor forms a reversible complex with ODC, as mammalian antizyme does.

Effects of exogenous polyamines on the decay of cellular ODC activity were examined. After 4 days of culture, spermidine was added to the medium at a final concentration of 0.2 mM, and total ODC activity, which was the sum of free and complexed ODC activities, was determined in the presence of excess amounts of DFMO-ODC. The total ODC activity decayed more rapidly after addition of spermidine than after addition of cycloheximide (Fig. 3A). Complex ratio [(total ODC-free ODC)/total ODC)] markedly increased after addition of spermidine (Fig. 3B). The accelerated decay caused by spermidine was partially blocked by simultaneous addition of cycloheximide, suggesting that protein synthesis was needed at least in part for this process. ODC decay

Table 2
Reactivation of ODC/inhibitor complex in crude extracts of Tn5 cells by DFMO-ODC

DFMO-ODC	ODC act. (U)				Inhibition (U)
	Extract 1	Extract 2	Extract 1 + Extract 2		
			Calculated	Observed	
−	7.55 ± 0.18	0.11 ± 0.01	7.66	5.25 ± 0.26	2.41
+	8.09 ± 0.27	2.79 ± 0.37	10.88	11.54 ± 0.55	
Complex (U)	0.54	2.68	5.63	6.29	

Crude extracts were prepared from Tn5 cells in which either ODC (Extract 1) or ODC inhibitor (Extract 2) had been induced as described in Section 2. DFMO-inactivated ODC (DFMO-ODC) was prepared as described in Section 2 and added in excess (27.6 U). The increases in ODC activity caused by addition of excess DFMO-ODC indicate the amounts of ODC/inhibitor complex (0.54 U in Extract 1 and 2.68 U in Extract 2). The ODC activity of combined extracts was smaller than the sum of ODC activity of each extract by 2.41 U. This represents the amount of free ODC inhibitor contained in Extract 2, which was supposed to form a complex with ODC of Extract 1 when the two crude extracts were mixed. Calculated amount of the complex of Extract 1 + Extract 2 (5.63) was figured out from the amounts of originally contained complex of Extract 1 (0.54) and Extract 2 (2.68) plus ODC inhibitor activity of Extract 2 (2.41) which should have formed a complex upon mixing the extracts. Values are mean ± S.D. of three experiments.

Table 3
Comparison of ODC inhibitory activities of rat antizyme and Tn5 inhibitor against Tn5 ODC and Rat ODC, respectively

Addition	Rat ODC (U)	Tn5 ODC (U)
none	2.55	1.26
Rat antizyme	0.12	0.56 [a]
Tn5 inhibitor	0.18 [b]	0.50

Rat antizyme was prepared as described in Section 2. Rat ODC was partially purified by DEAE-Cellulose. Rat ODC or Tn5 ODC was assayed with or without rat antizyme or Tn5 inhibitor.
[a] Four times as much rat antizyme was used for Tn5 ODC as for rat ODC.
[b] Four times as much Tn5 inhibitor was used for rat ODC as for Tn5 ODC.

was relatively slow upon addition of cycloheximide only, indicating that ODC of Tn5 cell was relatively stable under the condition (Fig. 3A). On the other hand, actinomycin D as such induced ODC in Tn5 cells, as reported in mammalian tissues and cells [27–30], and it did not affect significantly the decay of ODC caused by spermidine though there was a delay of one hour (Fig. 4). This delay seems to be due to ODC superinduction [27–30]. These results indicate that RNA synthesis is not needed for the destabilization.

Tn5 ODC was inhibited by rat antizyme and conversely rat ODC was inhibited by Tn5 ODC inhibitor (Table 3). But Tn5 ODC and Tn5 ODC inhibitor were not precipitated by rabbit antiserum raised against mouse ODC and rat antizyme, respectively (data not shown).

4. Discussion

Antizyme has been shown to be present in various vertebrates such as mammals, chickens, frogs and possibly eels [5,23,31]. The present results showed that an ODC inhibitor was induced by polyamines in insect-derived Tn5 cells. The inhibitor was likely to be a protein, since it was non-dialyzable and heat-sensitive. It inhibited ODC time-independently, indicating that it was not an enzyme like a protease. Instead, the inhibitor reversibly formed an inactive complex with ODC. These characteristics are consistent with that of mammalian antizyme. Unlike mammalian antizyme, however, the factor acted in a non-stoichiometric manner, suggesting that the binding between insect ODC and the inhibitory factor is relatively weak, compared with that between rat ODC and antizyme.

Insect ODC has been cloned from *Drosophila*. The primary sequence of *Drosophila* ODC has a clear homology to internal region of mouse ODC sequence, whereas both N-terminal and C-terminal regions lack homology [22]. The C-terminal region of mammalian ODC is known to be important for its instability. Upon truncation of C-terminal region of ODC, it becomes stable and gets out of the regulation by polyamines [32,33]. On the other hand, *Trypanosoma brucei* ODC, of which C-terminal region is truncated and is stable, becomes unstable when fused with mouse C-terminal region [34]. The C-terminal region of *Drosophila* ODC is 50 amino acids shorter than that of mammalian enzyme and lacks the degradation domain [22,32,33]. Furthermore, *Drosophila* ODC has no homology to antizyme-binding domain near N-terminus of mammalian ODC, which is necessary for negative feedback regulation of ODC by polyamines [22,35]. Lack of these domains in insect ODC suggested that insect ODC is a stable protein and its intracellular degradation is not regulated by polyamines. The present results showed that Tn5 cell ODC is relatively stable compared to mammalian ODC, but unexpectedly is destabilized by exogenously added polyamines. This destabilization is inhibited by cycloheximide but not actinomycin D, suggesting that a protein induced by polyamines at a post-transcriptional level is involved in the destabilization of insect ODC. The inhibitory factor induced by exogenously added polyamines has many common characteristics to mammalian antizyme as described above. These results suggest that in insect cells ODC is negatively regulated by polyamines through complex-formation with antizyme. Numbers of amino acids identical to antizyme-binding region of mouse ODC, which is composed of 24 amino acids, are 9, 12, and 13 in *Drosophila*, *Trypanosoma* and yeast, respectively [22,35,36]. *Trypanosoma* ODC can not bind to rat antizyme [35] and yeast ODC was not inhibited by rat antizyme (Murakami, Y., unpublished data). According to amino acid sequence of *Drosophila* ODC, insect ODC is expected not to bind to rat antizyme. Interestingly, however, insect ODC is inhibited

clearly, though weakly, by rat antizyme, and conversely, insect antizyme-like inhibitor inhibits rat ODC. Both proteins may bind to some domain homologous between rat and insect ODC other than antizyme-binding region of mouse ODC. The cDNA of the antizyme-like inhibitor should be isolated in order to examine its function and role in the regulation of ODC and polyamine metabolism.

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